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Genetic engineering of tumor cells to express MHC class and subsequent use of said cells for treatment of established and metastatic tumors has yielded promising results in animal models for treatment of breast cancer. It is widely believed that the vaccine efficacy is due to the ability of such tumor cells to present tumor-specific antigens to $CD4^+$ T helper cells which activate the immune system to eradicate tumors. Next generation cells-based vaccines will have enhanced antigen presentation capabilities to further stimulate the anti-tumor immune response. It has recently been proposed that MHC class II molecules physically localize to cell-surface microdomains, termed lipid rafts, to enhance antigen presentation. Furthermore, a correlation has been observed where cell-based tumor vaccines that have high levels of MHC class II in such rafts have higher efficacy than those with diminished or abolished levels of MHC class II in rafts. We propose to further target MHC class II molecules to lipid rafts to enhance the antigen presentation capabilities of tumor cell-based vaccines and than to use these modified vaccine cells for the treatment of established, metastatic disease in mouse models of breast cancer.

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Introduction

Genetic engineering of tumor cells to express MHC class II and subsequent use of said cell-based vaccines for treatment of established and metastatic tumors has yielded promising results in animal models for treatment of breast cancer (1-3). It is widely believed that the vaccine efficacy is due to the ability of such tumor cells to present tumor-specific antigens to $CD4^+$ T helper cells which activate the immune system to eradicate tumors (4, 5). Next generation cell-based vaccines will have enhanced antigen presentation capabilities to further stimulate the anti-tumor immune response. It has recently been proposed that MHC class II molecules physically localize to cell-surface microdomains, termed lipid rafts, to enhance antigen presentation (6). Further more, a correlation has been observed where cell-based tumor vaccines that have high levels of MHC class II in such rafts have higher efficacy than those with diminished or abolished levels of MHC class II in rafts (7). We propose to further target MHC class II molecules to lipid rafts to enhance the antigen presentation capabilities of tumor cell-based vaccines and than to use these modified vaccine cells for the treatment of established, metastatic disease in mouse models of breast cancer.

Body

Note: Text appearing in the original statement of work (SOW) is underlined

Task 1: Generation and characterization of MHC class II and cysteine substituted MHC class II positive mammary carcinoma cells, Months 1-12

a. Create point mutations in the DNA encoding the cytoplasmic domain of the beta chain of I-A^d that substitute cysteine residues for wild type amino acids and subclone, along with the alpha chain of I-A^d into a bicistronic expression plasmid (pIRES). Create similar expression vectors using wild type beta chain DNA.

Results- The strategy for creation of MHC II mutants was to first create mutations in either the alpha or beta cDNAs encoding the alpha or beta chains of the MHC II molecule I-A^d. Both cDNAs would be subsequently subcloned into a commercially available vector containing an IRES site to allow simultaneous expression of both cDNAs. The MHC II-IRES cassette would then be subcloned into a retroviral expression vector for the creation of a retrovirus that could transform target cells to express I-A^d. Two mutations have been created thus far and are shown in figure 1. The first contains two point mutations in the beta chain at non-essential amino acids where G226 and P228 have both been changed to cysteine. The resulting construct is termed A^d2cys. The second clone contains a four amino acid insertion at the transmembrane-cytoplasmic domain interface of the alpha chain. The amino acids cysteine-valine-arginine-cysteine have been shown to contain the raft localization sequence from the protein LAT (linker associated with T cell activation) (8) and therefore the second mutant is called A^dLAT. Both constructs have been successfully created and sequenced and subsequently cloned as described above to create retroviral particles for use in further experiments.

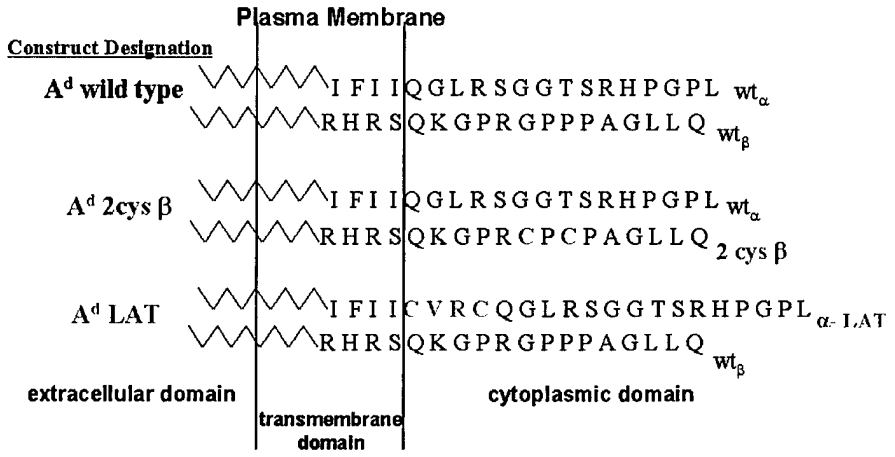

Figure 1. Proposed I-A^d constructs containing lipid raft favorable mutations. The drawings indicate the amino acid sequence of the wild type alpha and beta chains of the I-A^d MHC class II molecule. Proposed mutations are highlighted along with the construct name.

a. Transfect 4T1/CD80 mammary carcinoma cells with MHC class II expression plasmid from task 1a. Limit dilution clone to establish 3-5 clones of each transfectant.
b. Characterize expression of I-A^d on each cell line using flow cytometry. Check for positive expression of CD80 and endogenous MHC class I molecules.

Results- The mouse mammary carcinoma cell line 4T1, has been successfully transduced with both I-A^d constructs described in task 1 as well as the costimulatory molecule CD80. MHC II$^+$/CD80$^+$ cells were selected by a combination of drug resistance, magnetic bead sorting, fluorescent sorting, and subcloning. The cell lines to be used in further studies were analyzed by FACS analysis for expression and the data is summarized in figure 2.

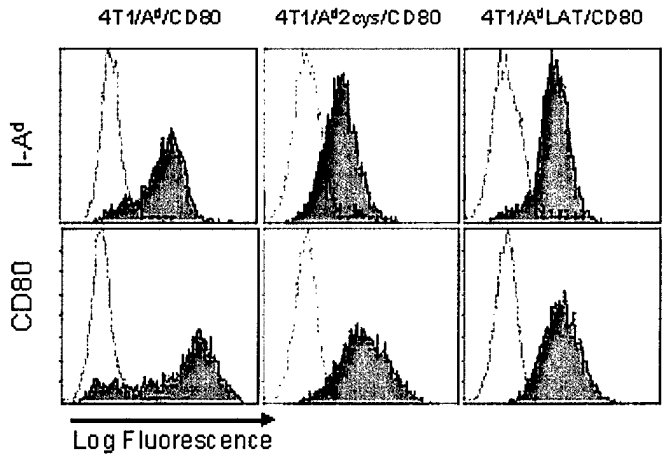

Figure 2. Expression of I-A^d and CD80 molecules on the surface of 4T1 cells. 4T1 cells were transduced with retrovirus particles containing CD80 and the appropriate I-A^d construct. Following a combination of drug selection, magnetic bead and fluorescent sorting, and limited dilution cloning, cell lines were characterized for I-A^d expression (top panel) and CD80 (lower panel) using flow cytometry. Shaded histograms were generated using an I-A^d or CD80 monoclonal antibody followed by an appropriate fluorescently-coupled secondary antibody while open histograms indicate the secondary antibody staining alone.

d. Assess MHC class II activity on transfected 4T1 cells by assessing stable dimer formation by western analysis. Test the ability of transfectants to present synthetic peptides to peptide-specific I-A^d-restricted T cells *in vitro*.

Results- Western analysis of SDS-stable I-A^d heterodimers has not been completed as of yet. To determine if mutant MHC II enhances antigen presentation, 4T1 cells expressing mutant MHC II were used as antigen presentation cells to T cell hybridomas. Cells were mixed with varying levels of the ovalbumin (OVA) peptide 323-339 and the T cell hybridoma DO11.10 which recognizes OVA in the context of I-A^d. Following overnight incubation, IL-2 release by hybridomas was quantified by ELISA. The data is summarized in figure 3 and suggests that enhanced raft localization does not increase antigen presentation capabilities of cells. These data do demonstrate that the A^d2cys construct still forms functional MHC II molecules capable of presenting antigen.

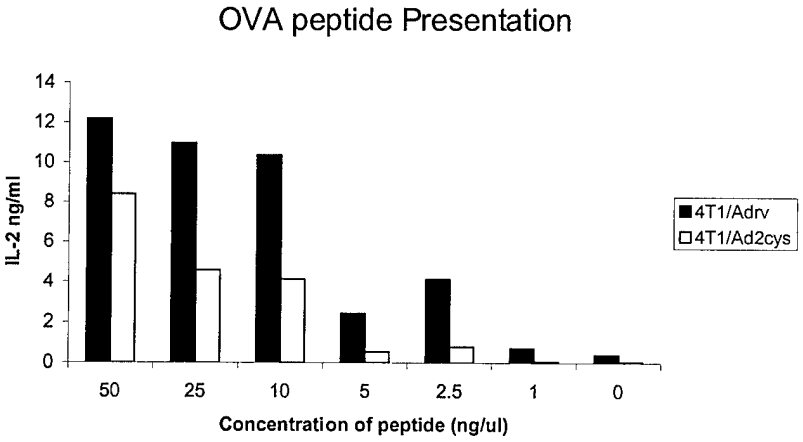

Figure 3. Antigenic peptide presentation by 4T1 cells expressing mutant MHC class II. Indicated concentrations of OVA peptide 323-339 were added to a mixture of indicated 4T1 cells and the T cell hybridoma DO11.10. After overnight incubation IL-2 release by T cells quantified by ELISA.

Task 2: Characterization of MHC class II-lipid raft interactions in transfected mammary carcinoma cells, Months 12-18.

a. Treat the wild type and cysteine substituted MHC class II positive 4T1 cells established in task 1 with a variety of detergents at different concentrations to isolate detergent insoluble lipid rafts. Quantify MHC class II content by trace densitometry following western blot analysis of both lipid raft and plasma membrane fractions of detergent treated cells.

Results- Mutant forms of MHC II were tested for their ability to localize to lipid rafts. Using the common detergent lysis and sucrose density flotation method for raft extraction, I-A^d cells were first labeled with biotin to label cell-surface proteins and subsequently lysed in a buffer containing 0.2% Triton X-100. The cell lysate was subjected to ultracentrifugation in a discontinuous sucrose gradient and rafts were isolated as detergent-

insoluble, low density material. Gradient fractions were probed for the raft-marker caveolin and the non-raft marker tubulin to identify raft and non-raft fractions respectively (data not shown). Fractions were then probed for I-A^d using an antibody that recognizes the beta chain of the molecule. Our initial results suggest that mutant forms of I-A^d better localize to lipid rafts in accordance with our hypothesis (figure 4A). Cell-surface MHC II was also assessed for raft localization. Cell-surface proteins from raft and non-raft fraction were isolated by precipitation with stretavidin-coupled sepharose. Precipitated proteins were analyzed by western blot for I-A^d. Our initial results suggest that at least one mutant A^d2cys has more MHC II in cell-surface rafts than wildtype I-A^d (figure 4B). These results are encouraging and suggest mutant MHC II may better localize to rafts; however, experiments need to be done with varying levels of detergent and using chemicals to disrupt rafts to confirm the presence of MHC II in rafts.

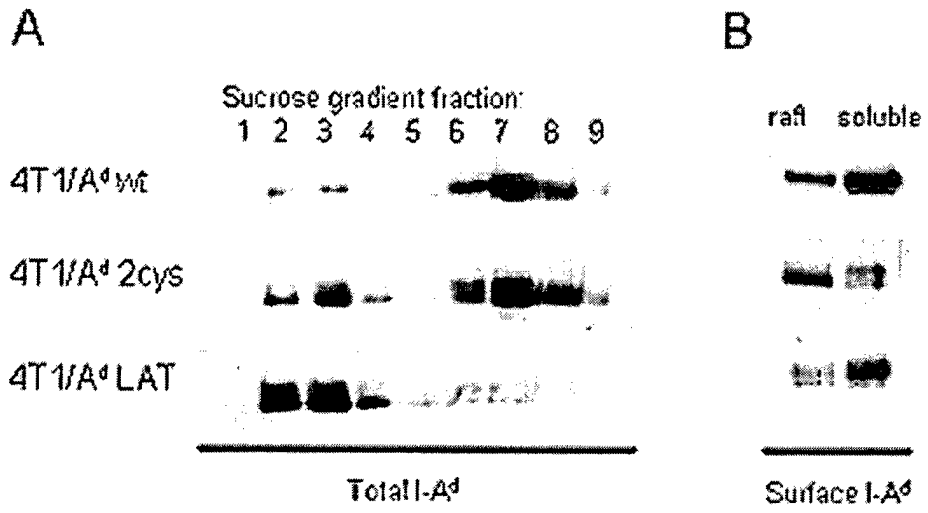

Figure 4. Localization of wild type and mutant MHC class II molecules to lipid rafts of 4T1 cells. *A*. Detergent lysed cell extracts were subjected to density gradient fractionation and probed for I-A^d by western blot. Fractions 2-4 correspond to the low-density raft fractions and fractions 7-9 as the soluble membrane fractions. *B*. Cell surface proteins were labeled with biotin prior to detergent lysis and, following raft isolation, subjected to precipitation by stretavidin-coupled sepharose. Precipitates were analyzed by western blot for I-A^d.

b. Determine palmitate incorporation into wild type and mutant MHC class II molecules by incubating cells with radio-labeled palmitate, followed by immunoprecipitation of MHC class II and measurement of incorporated palmitate.

Result- Experiment has not been attempted as of yet.

The following tasks remaining in the SOW have not been attempted yet:

Task 3: Examination of immunogenicity of non-mutated and cysteine-substituted MHC class II+ 4T1 mammary tumor cell vaccines, Months 12-30.

[**Note**: All animal experiments will be conducted 2-3 times using 8-12 animals/group. Experiments will use BALB/c female mice, 6-20 weeks of age.]

Task 4: Quantification of antigen presentation capacity of MHC class II+ and cysteine substituted MHC class II+ mammary carcinoma tumor cell vaccines, Months 18-36. [Note: Subsequent experiments will use TCR transgenic mice whose TCR recognizes OVA in the context of I-A^d. *In vivo* studies will be done using 8-10 mice/group and repeated at least once].

KEY RESEARCH ACCOMPLISHMENTS

- Lipid raft favorable mutations have been introduced into the MHC class II molecule I-A^d
- The mouse mammary carcinoma cell line 4T1 has been modified to express mutant MHC class II molecules. The levels of MHC class II and CD80 have been quantified
- Stable heterodimer formation of mutant MHC class II molecules has been confirmed using a peptide exchange antigen presentation assay
- Enhanced lipid raft localization of mutant MHC class II molecules has been observed

Reportable Outcomes

The data described above has been presented at the following meetings:

- The Keystone Lymphocyte Activation and Signaling Meeting- January 2004, Steamboat Springs, CO USA
- Experimental Biology (FASEB)- April 2004 Washington DC, USA

The following paper was also published:

- Dolan, B.P., et al., *Invariant chain and the MHC class II cytoplasmic domains regulate localization of MHC class II molecules to lipid rafts in tumor cell-based vaccines.* J Immunol, 2004. **172**(2): p. 907-14.

Conclusions

The goal of this project was to determine if MHC class II molecules could be targeted to lipid rafts and, if so, whether or not such a strategy would yield a more potent therapeutic agent for the treatment of metastatic disease caused by breast cancer using an established murine system. Thus far we have been able to generate mutant forms of MHC class II, express them in a mammary carcinoma cell line, and preliminary determine enhanced raft localization of such molecules. The next phase of the research will involve using these cell lines as therapeutic agents to treat metastatic disease and results should be generated within 1 years time. Further work will also help to identify the mechanism by which lipid raft localization effects vaccine efficacy.

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